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Invasion of *Pectinatella magnifica* in Freshwater Resources of the Czech Republic

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Abstract*—Pectinatella magnifica* (Leidy, 1851) is an invasive freshwater animal that lives in colonies. A colony of *Pectinatella magnifica* (a gelatinous blob) can be up to several feet in diameter large and under favorable conditions it exhibits an extreme growth rate. Recently European countries around rivers of Elbe, Oder, Danube, Rhine and Vltava have confirmed invasion of *Pectinatella magnifica*, including freshwater reservoirs in South Bohemia (Czech Republic). Our project (Czech Science Foundation, GAČR P503/12/0337) is focused onto biology and chemistry of *Pectinatella magnifica*. We monitor the organism occurrence in selected South Bohemia ponds and sandpits during the last years, collecting information about physical properties of surrounding water, and sampling the colonies for various analyses (classification, maps of secondary metabolites, toxicity tests). Because the gelatinous matrix is during the colony lifetime also a host for algae, bacteria and cyanobacteria (co-habitants), in this contribution, we also applied a high performance liquid chromatography (HPLC) method for determination of potentially present cyanobacterial toxins (microcystin-LR, microcystin-RR, nodularin). Results from the last 3-year monitoring show that these toxins are under limit of detection (LOD), so that they do not represent a danger yet. The final goal of our study is to assess toxicity risks related to fresh water resources invaded by *Pectinatella magnifica*, and to understand the process of invasion, which can enable to control it.

***Keywords*—**Cyanobacteria, freshwater resources, *Pectinatella magnifica* invasion, toxicity monitoring

I. Introduction

PECTINATELLA MAGNIFICA (Leidy, 1851) (*PM*) is a bryozoan species spreading with an invasive character. A native area of the animal is the east part of the Mississippi River, from Ontario to Florida, where it was observed already in 19th century. Since then, *PM* has been spreading also to Korea, India, Japan, and Turkey. In Europe, it has been recorded in Germany, Romania, Turkey, and France. Its occurrence in the Netherlands has been first reported in 2003. The newest discoveries are for the Rhine basin in the area between Luxembourg and Germany. Recently, it was also recorded in the Czech Republic [1].

A colony of *Pectinatella magnifica* is built from a gelatinous matrix covered by hundreds of individual filter feeding zooids. Owing to its massive occurrence, it presumably has an important influence on the ecosystem (species composition, trophic level, and hydro-chemistry). Large *PM* colonies can also clog water intake and irrigation pipes creating economic and engineering challenges. Therefore, the implication of *PM* invasion in the freshwater reservoir concerns not only biology but also ecology, planning of further usage of water supply resources and thus the quality of life.

The knowledge about chemistry of *PM*, about its second metabolites, and also the recognition of the state, development and plankton dynamics (which affects the food chain) of sandpits, ponds and rivers with *PM* colonies is a key for understanding and controlling the invasion.

In order to understand the invasion in a complex way, this paper consists of three separated parts concerning i/ statistical evaluation of *PM* spread during the season 2015, ii/ analysis of bacteria related to *PM* and microbiological activity of *PM* extracts, iii/ selected chemical analysis of lyophilized *PM* samples.

II. Statistical Evaluation of *Pectinatella magnifica* Colonies in Area Třeboňsko, 201 Seasons

A. Invasion of Pectinatella magnifica in Area Třeboňsko: Biomass Production

In the Protected Landscape Area and Biosphere reserve "Třeboňsko" the bryozoan was first found in 2003 in a mesotrophic sandpit. Then, the species gradually spread to many other sites on the Třeboňsko area and at most of these locations its occurrence has invasive properties [2].

The most important parameter affecting *PM* occurrence is the low trophicity of water, thus it expanded to some gravel sandpits and fishponds without intensive pisciculture. Colonies of this invasive bryozoan in this area are found typically on submerged branches of willow trees [3].

Biomass, expressed by weight and the number of colonies, is an important factor in terms of the potential production of biologically active substances. Higher biomass production means a higher amount of zooids, thus more metabolically active units of *Pectinatella magnifica*. In addition, a larger amount of biomass means higher volume of the matrix that is colonized by other potential producers of biologically active substances: algae, cyanobacteria and bacteria. Therefore, the monitoring of amount of biomass is one of the basic work for following studies.

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B. Statistical Methods

1) Data Collection Methods

Sample collection was carried out at five localities – two gravel sandpits of "Cep" and "Veselí I", and three ponds of "Podřezaný", "Hejtman", and "Nový Kanclíř". Six transects were chosen on each locality in the length of 10 m along the shore and of width 5 m. All colonies found in these transects were measured and weighted. Sample collection was duplicated on identical transects in July 2015 (first sampling) and August 2015 (second sampling).

2) Data Analysis Methods

The exploratory data analysis was performed by bar charts and the interference analysis by analysis of variance. A Kruskal-Wallis test was chosen, as the data were not normally distributed.

The localities were used as the independent variables, while the dependent ones were the biomass, number of colonies and the average colony mass. The null hypotheses were designed so, that the biomass/number of colonies/average colony mass do not differ between the colonies. The critical significance level was set equally for all analyses comparing the dependent variables to α=0.05. For comparing the dependent variables this significance level was adjusted following Bonferroni to α=0.02. Next, the H-statistics were computed and compared to the critical value $\chi 2$ — distribution with m-1 degrees of freedom. The null hypothesis was rejected, whenever the statistical value was higher than the value of $\chi 2$ — distribution with m-1 degrees of freedom.

C. Results

1) The Exploratory Data Analysis

Biomass

A total amount of biomass was higher for the first sample collection in July 2015 at all of the localities. On Hejtman, Cep and Veselí I, no colonies were found during the second collection and on Nový Kanclíř only one colony was found in August, weighing 1.7 kg. The reason for the lack of colonies in the second term of collection of 2015 were probably unusual climatic conditions (see Conclusions, part D).

The biggest biomass from the collection in July (129.4 kg) was found on pond Nový Kanclíř. Quite high level of biomass (compared to previous years) was found on the pond Podřezaný (409 kg). It was also considerably higher than the biomass found on other localities – Veselí I (41 kg), Hejtman (25 kg) and Cep (0.6 kg).

Colony Number

The highest number of colonies within the first collection was found on the gravel sandpit Veselí I (282), and on Nový Kanclíř (257), significantly lower on Hejtman (48) and the lowest on Cep (12). Within the second collecting in August, no colonies were found on three localities (Hejtman, Cep and Veselí I). On the pond Nový Kanclíř, only a single colony was found. Considerably higher amount within the second sampling was only found on Podřezaný pond (450 colonies in comparison to 258 in the first sampling).

Average Colony Mass

The average colony mass was calculated as a ratio of the cumulative mass collected in the six transects at a given locality divided by the total number of colonies counted there. The highest average colony mass from the first sampling was recorded on the pond Podřezaný (1.58 kg), huge colonies with the average weight of 1.08 kg were found also on Nový Kanclíř. The average colony mass on Hejtman was 0.52 kg, and the lowest on the gravel sandpit Cep (0.05 kg).

Average colony masses for the five localities in the season 2015 are plotted in Fig. 1 (white bars). Due to the lack of colonies in August 2015 on most of the localities, the second collection was not considered for the calculation of an average. For comparison, there adequate data from previous season (2014) were added to the graph (grey bars).

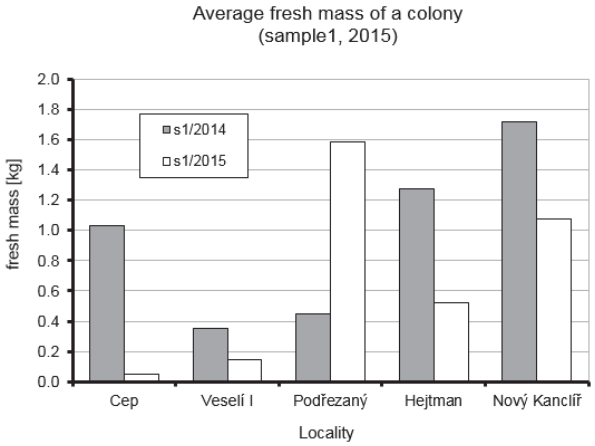

Fig. 1 Comparison of the average fresh mass of a *PM* colony at five localities in season 2015 (white bars) to the previous season 2014 (grey bars). The first two localities are gravel sandpits; the others are ponds. Due to extraordinary weather in summer 2015 (see text), instead of average values from two sampling dates, only the first sampling was considered.

2) Interference Analysis

Biomass

The null hypothesis was that the amount of biomass is the same on all localities. This hypothesis was rejected on a significance level α=0.05 (H=20.64, $\chi 2$=11.07). The amount of biomass differs greatly within the localities and specific locality has a big influence on the biomass production. After a simultaneous comparison of the localities on a significance level α=0.02, the same significant difference in the biomass production on all of the localities was found.

Number of Colonies

Data of this factor were the same as of the previous one. The null hypothesis that the number of the colonies is the same on all localities was rejected with the level of significance α = 0.05 (H=55.92, $\chi 2$=11.07) and with a simultaneous comparison of the localities on a significance level α = 0.02 a significant difference in the number of

colonies was found.

Average Colony Mass

The situation with this parameter is also the same as for the other parameters. That means a considerable dissimilarity between the average colony mass on the studied localities. The null hypothesis was rejected on the level of significance $\alpha = 0.05$ ($H=113.79$, $\chi 2=11.07$) as well as simultaneous comparison of the localities on a significance level $\alpha = 0.02$ found a significant difference in the average colony mass within all of the localities.

D. Conclusions

The season of 2015 varied from the previous ones primarily because of lack of colonies at the second sampling (August 2015) at three of five localities and because of a great increase of biomass on the pond Podřezaný in comparison with previous years. An explanation can be the extraordinary weather course: a period of high temperatures and drought caused an increase of trophicity in most freshwaters. The exception was a pond Podřezaný, into which less eutrophic water flows from the forest.

Preliminary statistical results showed dissimilarity of biomass production on different localities and also dissimilarity of biomass production between seasons 2015 and 2014. Study that is more detailed will be needed to correlate factors of *PM* invasive spreading within a longer time period.

III. Microbiota Associated with *Pectinatella magnifica* and Its Antimicrobial Activity

Because marine bryozoan are harbored by symbiotic bacteria which are known for the production of antimicrobial [4]–[5] and other bioactive compounds [6]–[7], there is a presumption that also freshwater *P. magnifica* may serve as a host for some specific bacterial species which may possess antimicrobial activity. Hence, the aim of our part of the study was to enumerate and isolate culturable bacteria from *PM* colonies and test their antimicrobial activity.

TABLE I
Bacterial Numbers Determined in *Pectinatella magnifica* colonies and water

Type of bacteria	Type of sample	No. of samples	Bacterial counts (log CFU/g or mL)	
			Varied from to	Median
Aerobic	Gelled mass	144	3.64 – 6.65	5.01
	Superficial layer	144	4.96 – 9.51	6.64
	Water	48	1.88 – 5.12	3.23
Facultative anaerobic	Gelled mass	144	3.00 – 6.26	4.53
	Superficial layer	144	4.91 – 9.20	7.51
	Water	48	1.20 – 3.63	2.42
Anaerobic	Gelled mass	144	1.00 – 6.85	3.65
	Superficial layer	144	1.74 – 7.90	4.15
	Water	48	1.00 – 2.96	1.75

A. Material and Methods

Samples of *PM* colonies and surrounding water were collected at four locations: two ponds "Hejtman" and "Kanclíř", two gravel sandpits "Cep" and "Veselí I". Three colonies from each location were collected at three different sampling times during the years 2012–2015. The superficial layer of bryozoan colonies containing zooids was collected separately from the inner gelled mass. Samples were aseptically transferred to vials containing oxygen-free peptone water, kept in a refrigerator and analyzed within 5 hours after collection. Water samples were collected into a sterile vessel and kept cold at 4 °C until analyzed.

Bacterial counts were determined by cultivation on yeast extract-tryptone agar (YT; Oxoid) supplemented with 1 g/L of glucose (yeast-tryptone-glucose agar; YTG). Samples were homogenized and serially diluted in peptone water under anaerobic conditions. One mL of appropriate dilutions was transferred to sterile Petri dishes, which were immediately filled with YTG agar. Cultivation was carried out at both, aerobic and anaerobic (Anaerobic Plus System; Oxoid) conditions at 25 °C for 3 days. Under the anaerobic condition, also facultative anaerobes were able to growth. So, for the determination of strictly anaerobic bacteria, neomycin was added to the media at a concentration 70 mg/L to avoid the growth of facultative anaerobes. After incubation, bacterial colonies were counted and data were expressed as a log CFU/g. Bacterial colonies representing a wide range of pigments and colony types were picked up from each cultivation variant, enriched in YTG medium and identified [8]. Bacteria in water samples were enumerated in triplicate using YTG agar, cultivation was performed under the same conditions as described above.

Antimicrobial activity of cell-free supernatants from *PM* associated bacteria against potential pathogens, meat-spoiling psychrophilic bacteria, intestinal bacteria and bacteria isolated from *PM* was tested by agar-well diffusion method. Cell-free culture supernatants were obtained from *Acinetobacter pitii* (2 stains), *Aeromonas veronii* (12 strains), *Aquitalea denitrificans* (10 strains), *Aquitalea magnusonii* (5 strains), *Enterobacter* sp. (3 strains), *Klebsiella* sp. (2 strains) *Lactococcus lactis* ssp. *lactis* (2 strains), *Leuconostoc pseudomesenteroides* (1 strain), *Pseudomonas* sp. (5 strains), and *Sphingomonas pituitosa* (2 strains) by microcentrifugation (18 000 x g, 4 °C, 20 min) of overnight bacterial cultures. 20 mL of nutrient agar was added to 1 mL of overnight cultures of potential pathogenic, meat-spoiling, intestinal and *PM* associated bacteria (tested strains: 5 strains of *E. coli*, 3 strains of *Salmonella* sp., 7 strains of *Clostridium* sp., 3 strains of *Lactobacillus* sp., 2 strains of *Bifidobacterium* sp., *Acinetobacter parvus* CCM7030, *Moraxella canis* CCM4590, *Pseudomonas aeruginosa* CCM1960, *Pseudomonas fluorescence* CCM2115, *Propionibacterium acnes* DSMZ183, *Serratia marcescens* DSMZ30212, *Micrococcus luteus* ATCC10240, *Listeria monocytogenes* ATCC7644, and 15 strains isolated from *PM* colonies). Plates were dispersed and agar allowed to firm. Antimicrobial tests were done under both, aerobic and anaerobic conditions and different cultivation media were used. The first one was the basic medium appropriate for each tested bacterium; the second medium was prepared from *PM* water extract, and the last one contained blood. Wells in the agars were created using a 6-mm

cork borer. Into the wells 100 μL of cell-free supernatant triplicate were pipette, plates were incubated for 10 hours in 4 °C to let supernatant diffuse to agar, and cultured 24–48 hours at appropriate temperature. Zones of inhibition were measured after cultivation.

B. Results and Discussion

Counts of culturable bacteria in *Pectinatella magnifica* colonies and the surrounding water are listed in Table I. In most cases, all three groups of bacteria were more numerous in the superficial structures of the bryozoan colonies (by one order for aerobic and anaerobic bacteria, in the case of facultative anaerobes by tree orders) compared to gelled mass. More numerous microbiota in superficial layers may be expected because this part contains zooids with simple U-shaped gut that obtains nutrients by water filtration. In both structures of *Pectinatella magnifica* colonies dominated aerobic bacteria with counts one order higher than numbers of facultative anaerobes, and two orders higher than anaerobic bacteria counts. The most variable numbers were obtained after anaerobic cultivation of both structures of *PM* colonies. No trends in the bacterial numbers at each individual locality during each year were observed. It was expected that the highest bacterial counts would be present in colonies sampled from water with the highest microbial contamination, but this was true only in some cases. It was shown that number of microorganisms, in both structures of *Pectinatella magnifica*, did not correlate with the number of microorganisms in surrounding water.

Only one strain of *Pseudomonas moraviensis* 16/12 and two strains of *Aeromonas veronii* 8/12 and 12/13 showed antimicrobial activity against human faecal clostridia. *Aeromonas veronii* 12/13 inhibited also the growth of *Pseudomonas moraviensis* 16/12. Diameters of inhibition zones varied between 8 and 16 mm. Neither of tested conditions nor media affected the antimicrobial activity of tested cell-free supernatants. The growth of other strains was not inhibited by tested bacterial supernatants. Bacteria producing inhibition zones did not decreased pH of cultivation media; so their antimicrobial activity was probably caused by other compounds than acids. Bacteria are well known for the production of many different antibacterial substances. Antimicrobial activity was described for some *Aeromonas* strains [9] and *Pseudomonas fluorescence* is producer of monoxycarbolic acid class antibiotic mupirocin, a mixture of several pseudomonic acids [10], [11]. Mupirocin is effective against aerobic Gram-positive bacteria but most of the anaerobes are resistant [12]. Mupirocin is used for the treatment of skin infections, methicillin resistant *Staphylococcus aureus* and for its activity spectrum is part of cultivation media for the detection of bifidobacteria in fermented and non-fermented milk products containing bifidobacteria and lactic acid bacteria simultaneously (ISO/IDF 220:2009).

IV. Chemical Analysis of Metabolites and Toxins Related to *Pectinatella magnifica*

Little is known about chemistry of *Pectinatella magnifica.* In 1930 Morse [13] has published preliminary results about composition of the jelly-like secretion of *PM* concluding that the gel is not a collagen-like polymer but rather a true protein which, together with extreme growth rate, he has considered as an interesting case of extremely rapid synthesis of proteins. A course of biuret reaction has been similar to albumins, the gel proteins have been heat-coagulable, and in the proteins the author has confirmed amino acids of tyrosine, tryptophan and cystine. Inorganic compounds of sodium chloride, calcium and, surprisingly, no phosphorus have been confirmed. In statoblasts, the author has presumed presence of common chitin since after hydrolysis glucosamine and galactosamine have been found. Dry mass content of 0.4% has been reported, though our measurements (area Třeboňsko) showed dry mass content up to 2.2% in several samples.

A. Preparative Chromatography and Identification of Content Compounds of Pectinatella magnifica

We worked with lyophilized *PM* collected in Třeboňsko area in seasons 2009-2015 [14]. We focused on content compounds isolated and identified by methods of TLC, HPLC, GC-MS, NMR.

1) Extraction of Lyophilized *Pectinatella magnifica*, Isolation and Identification Process

The extraction procedure was carried out on a large portion of lyophilized *PM*. Firstly, 90% methanol was added to the material. The process of extraction was repeated three times for 24 hours to ensure the quality of extraction. The solvent was each time used fresh. The extract was than filtered and concentrated using rotavapor. The residual water was removed by lyophilization.

The dry extract was dissolved again in 90 % of methanol and 10 % water and was extracted using separatory funnel with hexane in overall ratio 1:1 (v/v, 3×). The combined hexane portions were evaporated to give **hexane** portion of extract. The methanol portion was concentrated using rotavapor to remove large part of methanol. The residue was diluted with water and repeated extraction with chloroform (3 × 24 h) was used to obtain combined **chloroform** portion. The water part was then three times repeatedly extracted with ethylacetate. The combined ethylacetate extracts after removing of solvent on rotavapor yielded **ethylacetate** portion. The water from residue was removed by lyophilization and later **water** portion was yielded.

All fractions obtained in described extraction procedures were analyzed using HPLC and TLC method according to the previously published procedures [15], [16]. No extracts showed significant signal when analyzed using HPLC-DAD detection, we obtained visible spots on TLC only after spraying with sulphuric acid and heating.

Isolation

Hexane and chloroform portions showing the possibility to isolate pure compounds were selected to undergo separation

using the column chromatography and preparative TLC on silica. Column chromatography was carried out on Merck silica gel 60 (particle size 0.040-0.063 mm). TLC plates of Silicagel 60 F_{254} (Merck) (UV detection at 254 and 366 nm, and spraying will sulphuric acid and heating to 100 °C, respectively) were employed. Different combinations of solvents were used to formulate the mobile phases suitable for successful isolation of content compounds. Tested were many combinations of chloroform, ethyl acetate, hexane, acetone, benzene, and ethyl acetate with addition of formic acid in ratios of 50:50, 60:40, and 80:20 (v/v). Fractions obtained by elution with selected mobile phases were collected and combined according to the similarity. Analytical HPLC was performed on Agilent 1100 apparatus equipped with a diode-array detector and Dionex Ultimate 3000 equipped with UV-Vis detector, respectively. Several Supelco Ascentis Express HPLC columns were used for analysis (RP-Amide, C18, C8, F5, Phenyl, all 10 cm × 2.1 mm, 2.7 μm).

Identification

The isolated compounds were identified using MS and NMR analysis. The MS spectra were obtained via GC-MS analysis of corresponding fractions: fused silica column HP – 5MS 30 m × 0.25 mm coated with film of polymethyl (5% phenyl) siloxane stationary phase. Temperature program: 140 °C hold time 1 min, then increase to 290 °C at a rate 10 °C/min, hold time 16 min. Injector temperature 290 °C. Carrier gas helium, linear velocity 30 cm/sec. Injection volume 1 l, splitter injection, split ratio 1:10. MS conditions: transfer line temperature 280 °C, ion source temperature 200 °C, 70eV, positive ion mode, full scan mode, mass range 50-650 mu. NMR spectra for ^{1}H and ^{13}C analysis were recorded using a Bruker Avance 400 Ultrashield spectrometer operating at a frequency of 400 MHz (^{1}H). NMR spectra were acquired in methanol-d_4 at 298 K and in DMSO-d_6 at 296 K with TMS as an internal standard. The ^{1}H- and ^{13}C-NMR chemical shifts (δ in ppm) were referenced to the signal of the solvent [3.30 ppm (^{1}H) and 49.9 ppm (^{13}C) for methanol-d_4, 2.49 ppm (^{1}H) and 53.6 ppm (^{13}C) for DMSO-d_6]. The 1D NMR experiment and 2D NMR experiments (COSY, HMBC, and HSQC, NOESY and TOCSY) were used to assign the individual ^{1}H and ^{13}C resonances.

2) Results and Discussion

Two groups of chemical compounds were isolated by above described chromatographic procedures from the hexane fractions, and later identified using spectral methods: i/ derivatives of fatty acids: myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, ii/ derivatives of sterols: campesterol, cholesterol, stigmasterol, crinosterol, 7-oxo-sterol.

To isolate other content compounds represented by small molecular substances, we used, similarly to previous experiments, column chromatography, preparative TLC and semipreparative HPLC. TLC on silica was used to choose a mobile phase suitable for separation with sufficient resolution of selected spots. The TLCs were analyzed using UV and visible light or combination of this with detection with sulphuric acid. Extracts and fractions were analyzed using several HPLC methods. We used mobile phase for reversed phase chromatography composed of inorganic solvent (water, formic acid, ammonia) and organic solvent (methanol or acetonitrile) with different stationary phase types (C18, RP-amide, C8). The detection was based on UV, Vis or ELSD detection.

A sequence of methods, which is commonly successful for obtaining small molecular secondary metabolites from natural material, was adopted, but unfortunately without any significant yield of pure substance in amount suitable for the identification and evaluation of biological activity. There are four main hypotheses, which can explain the lack of success of the isolation: 1) in general *P. magnifica* does not produce substantial amounts (below our limits of detection) of secondary metabolites; 2) because the mass of *P. magnifica* is mainly formed from a water rich gel matrix, which is not involved in production of content compounds, the amount of extractible compounds from the material obtained by lyophilization is too low in comparison with extracts obtained typically from natural material (e. g. plants); 3) characteristic compounds present in *P. magnifica* are unstable and conditions used for lyophilization and/or extraction can cause their decomposition; 4) the content compounds produced by *P. magnifica* are of completely different character (e. g. proteins) and thus they are not extractable/detectable by the methods applied.

B. Determination of Cyanobacterial Toxins in P. magnifica by HPLC

Cyanobacteria were found in the *PM* colony gel (typically of genus *Pseudanabaena, Komvophoron, Phormidium, Leptolyngbya*) [17]. Their number increases with the colony lifetime as often indicated by the gel colour.

Microcystins (MCs) and nodularins (NODs), hepatotoxins belonging to a diverse group of cyclic oligopeptides produced by cyanobacteria, where they have been confirmed and determined [18], [19]. Over the last few decades, MCs and NODs have become a serious ecological and health issue due to the massive cyanobacterial water blooms that have developed in eutrophied waters worldwide [18]. The toxicity of and risks from some MC variants have been studied in detail [20], [21], and the World Health Organization recommends a provisional guideline 0.001 mg/L of MC-LR for drinking waters [22]. Therefore, we adapted a published HPLC method [18], [19] in order to monitor these highly toxic compounds.

1) Experimental (Chemical and Methods)

Microcystin-LR, microcystin-RR and NOD were purchased from DHI LAB Products (Hørsholm, Denmark). Acetonitrile, water and methanol of HPLC grade and also formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC system was Dionex Ultimate 3000 (Dionex, USA).

Samples were lyophilized biomass from seasons 2009-2014 collected in Třeboňsko area. Each sample was ultrasonicated

for 15 minutes in a water-methanol mixture 1:1 [18], 10 minutes centrifuged and then filtrated (0.45 μm filter) into an HPLC vial.

2) Results

The results of the method transfer are shown in Table II. Typical chromatogram of a mixture of standards is shown in Fig. 2.

TABLE II
EXPERIMENTAL CONDITIONS OF THE ORIGINAL AND TRANSFERRED HPLC METHODS

	Babica *et al.* [18] Blahová *et al.* [19]	This paper
Stationary phase	Supelcosil ABZ+Plus 150x4.6 mm, 5 μm	Ascentis Express RP-amide, 150x2.1 mm, 3 μm
Mobile phase	acetonitrile+TFA*/ water+TFA, flowrate 1.0 mL/min, 30°C	acetonitrile+FA** / water+FA, flowrate 0.5 mL/min, 25°C
Gradient time	30 min	18 min
Detection	UV 238 nm	UV 240 nm
LOD	0.02 mg/kg dry mass	1.3 mg/kg dry mass
Retention time of MCs	11-17 min	3-6 min

* TFA = 0.1% trifluoracetic acid
** FA = 0.1% formic acid

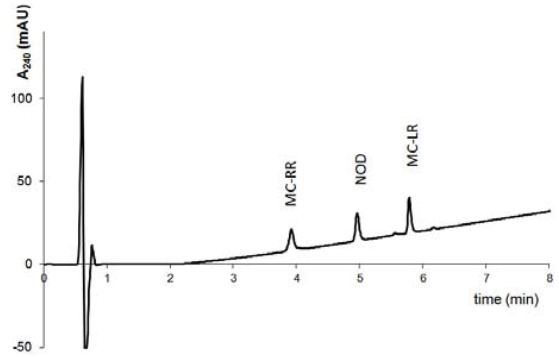

Fig. 2 Chromatogram of a standard mixture of nodularin (NOD) and microcystins RR (MC-RR) and microcystin-LR (MC-LR). The drifting baseline for given wavelength (240 nm) is due to gradient elution. For experimental conditions, see Table II, the rightmost column

3) Estimation of a "Real" LOD on a Model Case of Veselí I

The way of sample preparation (lyophilization of *PM* biomass) means that the calculated LOD relates to a dry mass of *PM*. However, this value does not show a real concentration of the toxins in the water reservoirs.

We tried to estimate LOD in the water (real environment) on a model reservoir with average production of biomass (Veselí I). This number is also a threshold concentration in water that could be determined by the method analyzing dry mass of *PM* after calculation with total water volume of the pond and also to the water volume around the pond bank.

Let us approximate the reservoir with a spherical cap having surface area 240,000 m^2, i.e. periphery of the circle is 1,750 m, radius of 275 m, maximum depth 4.5 m (average depth 3.5 m), and volume of 540,000 m^3. *PM* usually appears within 5 m from the pond bank. Within a strip (transect) of 10 m (i.e. 50 m^2), 30 kg of fresh biomass was collected. Thus, considering the whole periphery, there is 5,250 kg of fresh biomass in the reservoir. If 99% of it is water, we get the amount 52.5 kg of dry mass per 540,000 m^3 of water.

LOD was determined as 1.3 mg/kg of dry matter, which corresponds to 70 mg of toxins per 540,000 m^3, which is equivalent to 0.13 ng/L in water.

We can also estimate a maximum threshold concentration of toxins with the outer circle 5 m from the bank in depth of 1 m (the typical occurrence of *PM*): the volume of water is 8,600 m^3, thus the concentration in water will be 8.2 ng/L.

C. Mupirocin Occurrence in Bacteria Related to Pectinatella magnifica

Mupirocin (Bactroban or Centany) is an antibiotic of the monoxycarbolic acid class. It was originally isolated from *Pseudomonas fluorescens* NCIMB 10586 [10]. Mupirocin is bacteriostatic at low concentrations and bactericidal at high concentrations. It is used topically and is effective against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). It is known that mupirocin is a mixture of several pseudomonic acids [23]. To confirm mupirocin in the samples, an HPLC method [23] was adapted to our conditions.

1) Samples

Supernatants and supernatant dry matters samples were prepared parallely from both *Aeromonas* sp. and *Pseudomonas moraviensis* strains (see *A*, Section III) to be analyzed for presence of bioactive mupirocin. The supernatant dry matter obtained by lyophilization was extracted by methanol. In methanol insoluble portion was treated separately (2x2 samples). The methanol portion was subsequently extracted using separatory funnel to obtain hexane, chloroform, ethyl acetate, and water parts, similarly to *P. magnifica* biomass processing (2x4 samples) (see *A* 1, Section IV).

2) Experimental

Column was Supelcosil LC-8 (Supelco), 5 μm, 150 x 4 mm, mobile phase was acetonitrile with buffer (100 mM ammonium acetate adjusted with acetic acid to pH=4.0), detection @240 nm, flowrate was 1 mL/min, temperature was 25°C. Solvents of HPLC grade, ammonium acetate and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mupirocin was purchased from Applichem (Darmstadt, Germany) No. A4718 (90%).

3) Results

The experimental parameters of the adapted method were optimized as follows: the chromatographic column was Supelcosil LC-8, 150x4 mm, 5 μm; two mobile phases were used – i/ for isocratic elution of the mupirocin standard (70% acetonitrile with 30% of 100 mM ammonium acetate buffer, pH=4.0), ii/ for gradient elution of samples of bacteria extracts (10–60% of acetonitrile in 25 minutes with the same buffer). The flowrate was 1.0 mL/min, temperature was 25°C, UV-detection @240 nm. Estimated LOD was 0.2 mg/L. Applying

the optimized method isocratic method (run time of 11 minutes, retention time of mupirocin=pseudomonic acid A was 10 min) we confirmed that the commercial substance mupirocin is not a chromatographically pure compound (two extra peaks at 6.5 min and 7.5 min separated with resolution 1.0). In all the samples analyzed, mupirocin was below LOD.

D. Cytotoxicity of Extracts from Pectinatella magnifica

The main aim of the project is to assess potential toxicity of environment invaded by *PM*. Although there is not yet a particular toxic compound related to *PM* occurrence, fractions of lyophilized biomass of *PM* were tested for cytotoxicity.

1) Materials and Methods

Pectinatella magnifica extracts for *in vitro* tests were prepared and supplied by the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (see *A1*, Section IV). For the purpose of cytotoxicity evaluation, we used THP-1 cell line (European Collection of Cell Cultures, Salisbury, UK), cultured in RPMI 1640 medium (Verviers, Belgium) supplemented with 10% fetal bovine serum, 2% L-glutamine, 1% penicillin and streptomycin at 37 °C with 5% carbon dioxide. All reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Cytotoxicity of *PM* extracts was determined using a LDH assay kit (Roche Diagnostics, Mannheim, Germany) as described previously [24].

For 24 h the THP-1 cells were treated with a various extract concentrations ranging from 10 to 1000 µg/mL in RPMI 1640 medium (concentrations were selected according to [25]).

2) Results

For all tested extracts (PM1 – methanolic extract, PM2 – hexane fraction, PM3 – chloroform fraction, PM4 – ethylacetate fraction, PM5 – aqueous phase), the cytotoxicity was evaluated as a Relative cytotoxicity (relative to control values). Subsequently, from a dose-response curve we derived LD50 values for each of five extracts prepared from *Pectinatella magnifica*. As our results shown, cytotoxicity expressed as LD50 of the following *PM* extracts increased as follows: PM5 (250 ug/mL) > PM2 (75 ug/mL) > PM3 (40 ug/mL) > PM4 (31 ug/mL) > PM1 (29 ug/mL). According to [26] the treatment with *PM* extracts led to toxic effect on THP-1 cells, as their LD50 values were assessed to be <1000 µg/mL.

3) Conclusion

Results from the cytotoxicity assays demonstrated that extracts prepared from *Pectinatella magnifica*, exerted some toxic effect to cells *in vitro*. However, aqueous phase (PM5), which comes into consideration as the most common in nature did not show cytotoxicity. From these results, further studies on *Pectinatella magnifica* should aim its potentially negative effects to the environment.

V. Conclusions

Several points of view were examined related to invasion of *PM* in the area of Třeboňsko. Statistical analysis of *PM* biomass revealed that the invasion of *PM* (spreading to localities) is neither significantly related to a specific locality nor environment. The amount of produced *PM* biomass is very variable among localities and also years (seasons).

PM colonies examined in this study were colonized by bacteria from the environment. However, their abundance was higher than in surrounding water, so their symbiotic relationship with *PM* should not be excluded. Antimicrobial effect of three strains was demonstrated, but the substance responsible for this activity was not identified yet. Related bacterial are known for the production of antibiotic mupirocin, but its presence above LOD in our strains was not proven.

There is no apparent toxicity related to microcystins and nodularin (their concentrations are below LOD of our HPLC method), neither cytotoxicity related to aqueous extracts from *PM*.

We can conclude that the invasion of *PM* in water reservoirs of the Czech Republic does not represent a toxicity risk in this moment. However, a longer-term monitoring is needed.

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